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Award Number: W81XWH-12-1-0173

TITLE: Probing Tumor Microenvironment with In Vivo Phage Display

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REPORT DATE: July 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2013	Annual	01 July 2012 – 30 June 2013

4. TITLE AND SUBTITLE
Probing Tumor Microenvironment with In Vivo Phage Display

5a. CONTRACT NUMBER
W81XWH-12-1-0173

5b. GRANT NUMBER
W81XWH-12-1-0173

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Sanford Burnham Medical Research Institute
10901 N. Torrey Pines Road
La Jolla, CA 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this study is to develop specific probes that target various cells populations in the tumor microenvironment. Tumor stroma contains many cell populations, such as vascular endothelial cells, immune cells, mesenchymal cells, and extracellular matrix, which are critical to tumor development and progression. Although various probes have been developed for tumor vasculature, there is a scarcity of markers for tumor macrophages, fibroblasts, nerve cells and the matrix. The goal of our group is to make technical improvements in our phage display system, and find peptides that target carcinoma-associated fibroblasts (CAFs) in breast tumors. To reach the goal, we have improved our phage display technology by involving in the screens iRGD, a tumor-specific tissue-penetrating peptide. iRGD enhanced the penetration of co-applied phage libraries into breast tumor tissue by two fold, allowing the libraries to reach and probe the stromal compartments within the tumors. We have also optimized high throughput sequencing for phage DNA, and methods to isolate CAFs from breast tumor tissue. Multiple phage library screens are underway. In parallel, we have made an unexpected discovery that iRGD itself is an efficient CAF-targeting peptide, and that the iRGD receptor neuropilin-1 is a potential CAF marker in breast tumors. iRGD in combination with novel CAF-targeting peptides may result in an efficient probe for breast tumor imaging and therapy.

15. SUBJECT TERMS
Carcinoma-associated fibroblast; phage display; tumor-penetrating peptide; homing peptide; breast tumor.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION

Tumors grow in an environment that greatly contributes to tumor development and progression. Much attention has been paid recently to tumor vasculature, which has become an important target for tumor therapy. In addition to the vessels, the stromal elements in tumors include immune cells (macrophages polymorphonuclear neutrophils, lymphocytes, dendritic cells), mesenchymal cells (fibroblasts, mesenchymal stem cells), and extracellular matrix. Host-derived stromal cells evolve in parallel with carcinoma cells and can comprise more than half of the cellular content of a tumor. Each of these cell types plays an important role in determining the tumor formation and progression (or conversely, regression). An example is the production of various growth factors and cytokines by tumor macrophages, which can promote tumor cell growth and angiogenesis (Pollard, 2004; Chen et al., 2005). Crosstalk between tumor cells and tumor fibroblasts also promotes tumor growth, and these activated fibroblasts produce extracellular matrix that sometimes becomes so dense that it blocks the access of anti-tumor drugs to tumor cells (Kalluri & Zeisberg, 2006; Sund & Kalluri, 2009). Tumor vessels are well known to contain a multitude of markers not expressed or expressed at much lower levels, in normal vessels (Ruoslahti et al., 2010). Other stromal cells in tumor environment are also thought to be in an activated state and selectively express markers not present in their normal counterparts. However, in contrast to tumor endothelial cells there is a scarcity of markers for tumor macrophages, fibroblasts, and the matrix. Such markers will make it possible to selectively deliver compounds to the stromal elements and tumor stem cells for therapeutic, imaging and research purposes.

We are using new tumor-penetrating phage screening technology to identify tumor-specific peptides that bind to the non-vascular stromal elements in breast cancers. As our method can also probe tumor cells, we are also using the screens to identify markers for breast cancer stem cells. The method is based on co-administration of a phage library with iRGD, a tumor- penetrating peptide that facilitates penetration of co-administered compounds, including phage, into extravascular tumor tissue (Sugahara et al, 2009; 2010).

Body:
The *Specific Aims* approved for the study are:

Aim 1. Perform phage library screenings to identify homing peptides for individual cell types in tumors by using new tumor-penetrating screening technology

Task 1a. Perform tumor-penetrating screens for tumor-homing peptides on orthotopic and MMTV-PyMT *de novo* breast tumors. (Months 1-12)

Responsible PI
Kazuki Sugahara: Screens on carcinoma-associated fibroblasts
Erkki Ruoslahti: Screens on cancer stem cells and tumor-associated macrophages

Task 1b. Perform tumor-penetrating screens for tumor-homing peptides in human breast cancer explants. (Months 1-12)

Responsible PI
Kazuki Sugahara: Screens on carcinoma-associated fibroblasts
Erkki Ruoslahti: Screens on cancer stem cells and tumor-associated macrophages
Sarah Blair: Collection of human breast tumor explants

Task 1c. Optimize and validate the experimental approach and custom-made bioinformatics software for high throughput phage sequencing. (Months 1-12)

Responsible PI
Kazuki Sugahara

Aim 2. To validate the homing specificities of individual phage and synthetic peptides from Task 1 in ex vivo and in vivo tests

Task 2a. Analyze the homing specificity of homing peptides recognizing tumor fibroblasts. (Months 13-24)
Responsible PI
Kazuki Sugahara

Task 2b. Analyze the homing specificity of homing peptides recognizing tumor-associated macrophages (Months 13-24).
Responsible PI
Erkki Ruoslahti

Task 2c. Analyze the homing specificity of homing peptides recognizing cancer stem cells (Months 13-24).
Responsible PI
Erkki Ruoslahti

Summary

We have developed three tumor models in mice for the project and have acquired fresh human breast cancer samples from our clinical collaborator. We have shown using these tumors that the tumor-penetrating peptide iRGD does induce entry of phage into the extravascular tumor tissue as well as penetration of the phage into fresh tumor implants. These results validate our proposed screening approach. We have also established cell lines representing the various cell types we are targeting in tumors and developed methods for the isolation of these cells as primary cells from tumors. We are using the cells to enrich the libraries for phage clones that are capable of binding to the target cell prior to *in vivo* screening and are now conducting *in vivo* screens. Finally, we have set up high throughput sequencing for phage inserts, which will greatly increase the information obtained from the screens and speed up the process.

RESULTS

Aim 1. ***Perform phage library screenings to identify homing peptides for individual cell types in tumors by using new tumor-penetrating screening technology***

General

Tumor models. We have created a breast tumor mouse model by orthotopically injecting MCF10CA1a human breast cancer cells into nude mice. The MCF10CA1a tumors contain high amounts of tumor stroma, which iRGD efficiently penetrates (refer to Fig. 5). We have also bred MMTV-PyMT mice to have enough animals for screens and subsequent characterization assays. We have also collected a number of human breast tumor samples with help of our collaborator, Dr. Sarah Blair at the University of California, San Diego. We have collected 5 primary tumors, 2 metastatic lymph nodes, and 2 normal breast tissues.

Tumor-penetrating in vivo phage screening. Our hypothesis was that we would be able to probe extravascular tumor space with phage libraries by co-administering the library with iRGD. We have produced data supporting this notion and that the perfusion system we designed to focus the screening to the tumor interior works as intended. We administered insert phage (insert G7) to tumor mice with and without accompanying injection of iRGD. The mice were perfused with PBS (to remove blood), an acidic pH 2.5-buffer (to inactivate phage that had not been taken up by cells) and with a detergent solution (to remove the endothelial cell layer). A 3-5-fold higher titer of phage was recovered from the tumors of the mice injected with iRGD than the controls (Fig. 1),

showing the iRGD (and perfusion) effect we expected. These methods are now being used in ongoing *in vivo* screening.

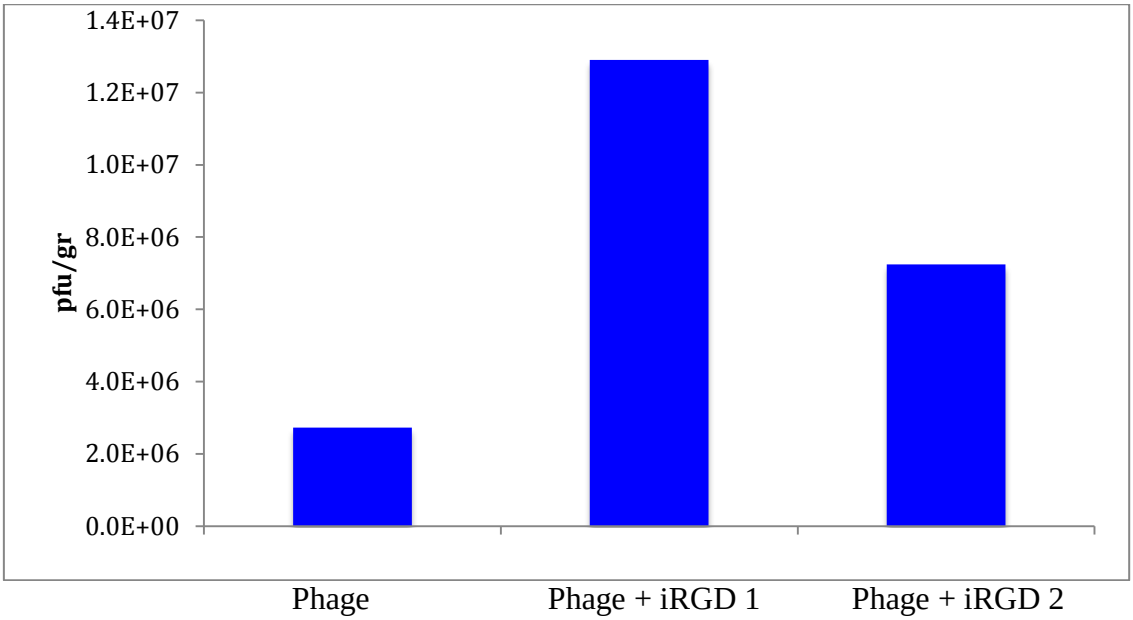


Figure 1. Effect of iRGD on the entry of phage into tumor tissue. Phage encoding an inert 7-glycine insert (10^X PFU was injected into transgenic breast cancer mice bearing tumors. The mice had received an injection of 100 micrograms of iRGD peptide 15 min earlier or served as a control. Thirty min after the phage injection, the mice were perfused through the heart as explained in the text, and the tumors were collected. Shown are the titers recovered from the tumors; iRGD promoted phage penetration 3-5 fold.

1a1. *Screens on carcinoma-associated fibroblasts*

We enrich phage for clones that are capable of binding to the desired target cells by *in vitro* phage panning prior to screening *in vivo.* We have acquired a number of cell lines for this purpose. For the targeting of tumor fibroblasts (CAFs), we have immortalized human breast CAF lines, hb6008 and hb6011. The two lines have been labeled with mCherry to allow isolation of the cells from tumors made by co-implantation of the CAFs and breast tumor cells (Fig. 2).

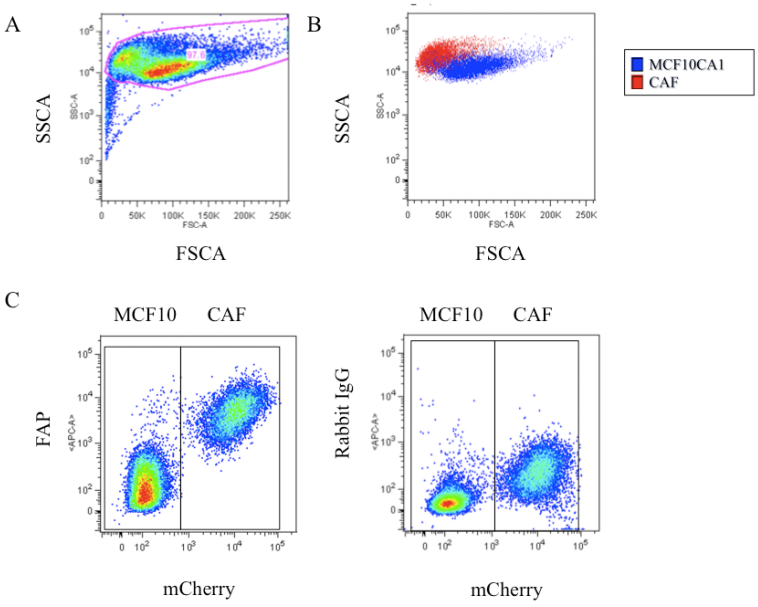


Figure 2. Separation of CAFs from MCF10CA1a breast tumor cells. MCF10CA1a human breast tumor cells and mCherry-labeled hb6011 CAFs were mixed *in vitro*, and the CAFs were separated based on mCherry expression using flow cytometry. (A) Forward (FSC) and side (SSC) scatter plot of the mixed population. (B) FSC and SSC plot showing the two different populations CAFs in red and MCF10CA1 in blue based on the mCherry gates plotted in (C). (C) Dot plots showing mCherry expression on the x-axis and fibroblast activation protein (FAP) or rabbit isotype control staining in the y-axis. Note that CAFs are potentially distinguishable using FSC and SSC alone.

We have also established a method to isolate CAFs from breast tumor explants. Tumors are chopped into small pieces with surgical blades, and treated with collagenase for 45 minutes. The crude cell suspension is washed in culture media, passed through a cell strainer, and CAFs are isolated on a magnetic column or by flow cytometry-based cell sorting using an antibody against fibroblast activation protein (FAP). During the optimization steps, flow cytometry revealed that 30-40% of total cells in a MCF10CA1a tumor are CAFs (refer to Fig. 7).

In vitro phage panning using a cyclic peptide library of the structure CX_7C (C=cysteine; X = random amino acid) on the hb6008 and hb6011 cells have been carried out. The emergence of phage clones expressing peptides that contain breast tumor homing motifs (e.g., **CREKA**SGSC; Simberg et al., 2007) shows that the panning is working. The phage pool enriched for breast CAF binding *in vitro* will be used as a starting material for subsequent *ex vivo* and *in vivo* screens. In parallel with the *in vitro* library enrichment process, we have started *ex vivo* and *in vivo* screens.

1a.2. *Screens on cancer stem cells and tumor-associated macrophages*

We have acquired human breast cancer cell lines that are highly enriched in tumor stem cells for in vitro phage panning. Combining spheroid culture, the stimulatory effects of extracellular matrix (Boudreau et al., 1995; Frisch and Francis, 1994; Petersen et al., 1992) and the differentiation inhibitory conditions of low oxygen atmosphere (Gustafsson et al., 2005; Helczynska et al., 2003) allow epithelial tumor-initiating cells to be amplified in culture in a highly enriched state. These cells are considered cancer stem cells because of their potent tumorigenicity and ability to differentiate to luminal and basal mammary cell lineages both *in vivo* and in culture distinguish them from most cancer cell lines, which are only capable of forming tumors of limited heterogeneity. Dr. Robert Oshima at our institute has used these methods to develop a breast cancer cell line (HCI-002) that is highly enriched in tumor-initiating (stem) cells (Castro et al., 2013). The Oshima laboratory has also established the MDA-MB-468, a human breast cancer cell line derived from a triple negative tumor, as one with a high content of tumor-initiating cells.

Initial screens with the HCl-002 showed that it was not possible to grow enough of these cells for the screens. We therefore switched to the MDA-MB-468 cells, which are easier to grow. We have used these cells to enrich phage for cancer stem cell binders. Figure 3 and Table 1 show examples of the results from such experiments. *In vivo* screens on Transgenic mice Tg(c3-1-Tag)cJeg/JegJ bearing breast cancer tumors using the enriched phage pool from the MDA-MB-468 cells showed RGD as the only repeated motif in limited conventional sequencing (Table 2). The prevalence of the RGD sequences as such, and in the context of a CendR motif (RGDK/R; Teesalu et al., 2009; Sugahara et al., 2009; 2010), creates a problem, because we know that these peptides are not going to be tumor stem cell-specific and obscure the presence of other potentially stem cell-specific sequences. To circumvent this problem we have initiated new screens in the presence of an excess of soluble RGD as an inhibitor that will prevent the binding of these sequences in the cell panning.

As described below, we have recently been successful in adapting ion torrent sequencing to T7 phage (a far more demanding project than we had anticipated). The high-throughput sequencing, which is underway, is likely to be more informative and will greatly facilitate the identification of useful homing peptides as well as broaden the array of such peptides recovered.

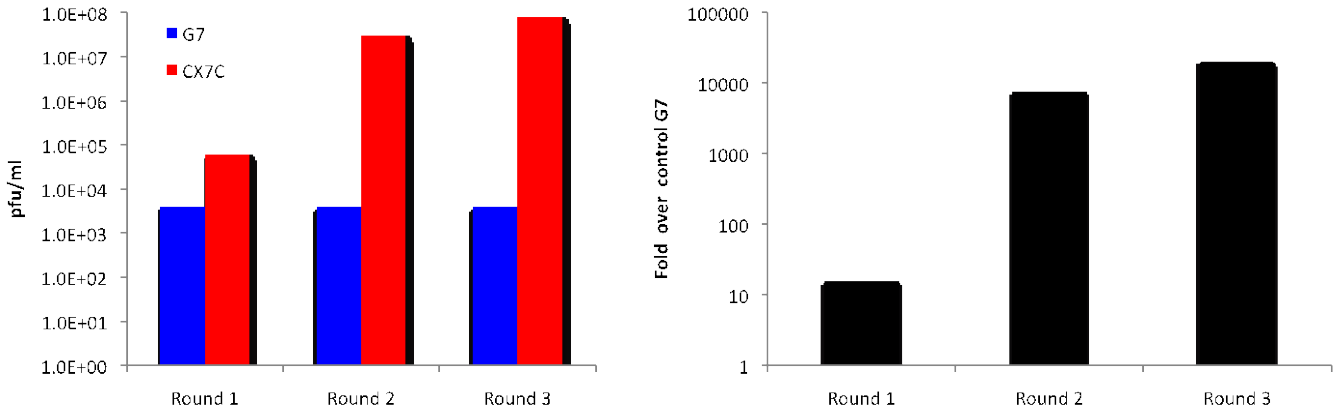


Fig. 3. Phage enrichment in *in vitro* panning. Two T7 phage libraries with the general structures of G7 (seven glycine residues; negative control), and CX7C (a cyclic peptide library), each 1x10^10 plaque-forming units, were incubated with 10^6 MDA-MB-468 cells, the cells were washed, and cell-associated phage were rescued for *in vivo* screening.

Table 1. Peptide sequences from round 3 of *in vitro* phage panning on cultured MDA-MB-468 cells*

1	CRGDRKGRC	41	CRSRNPRPC
2	CRGDLKGKC	42	CGRGDRRAC
3	CGRTEELDC	43	CRGDRAGRC
4	CGRGDQKRC	44	CRRWGDSTC
5	CRGDRKGKC	45	CRRRGDAAC
6	CRRGDKATC	46	CRGDRRGAC
7	CKQRGDLKC	47	CLKDGRSRC
8	CRGDRTGKC	48	CRGGWAAKC
9	CRGDRKGIC	49	CKRRGDQAC
10	CGRGDLKRC	50	CGRGDMRKC
11	CTRRGDRGC	51	CLSGGRPKC
12	CRGDRKGKC	52	CGRRGDRAC
13	CGRGDKPKC	53	CLSGGRPKC
14	CGRGDLGVC	54	CVSEGRPRC
15	CGRGDSLKC	55	CVKRGDSLC
16	CLRDGRPKC	56	CRGDSAAAC
17	CMRGE	57	CVKDGRPSC
18	CHVSRK	58	CSRGDLKRC
19	CRGDLRGKC	59	CRRGDRANC
20	CRGDLKKRC	60	CGRGDRRSC
21	CVADGRPKC	61	CRGDRLRNC
22	CNRRGDRAC	62	CRGDRLSNC
23	CRGDKTTNC	63	CLSDGRKRC
24	CRRGDKMKC	64	CRGDRAGRC
25	CARGDKKMC	65	CRRRGDAAC
26	CRGDRRLDC	66	CRRRGDKPC
27	CRGDKVPGC	67	CRGDSALKC
28	CRGDKGTEC	68	CKRRGDMAC
29	CRGDMGKRC	69	CGSKRETRC
30	CLADGRAKC	70	CGRGDMRKC
31	CISAKGKPC	71	CGRGDKARC
32	CLSDGRKRC	72	CKRR
33	CRRDDMTNC	73	CRVRRDRSC

34	CSRGDKRKC	74	CLADGRSKC
35	CRGDKGPDC	75	CSRREDKAC
36	CRGDSALKC	76	CRKRGDSPC
37	CMRGDKRRC		
38	CSKNEKQRC		
39	CRGDKAKHC		
40	CGRRGDLKC		

*The cyclic CX7C library was used in this experiment. Note the high frequency of peptides containing the integrin-binding RGD motif, often in the same context as in iRGD (iRGD sequence = CRGDK/RGPDC). An RXXR, so-called CendR motif creates a potential tumor-penetrating peptide (Teesalu et al., 2009; Sugara et al. 2009; 2010).

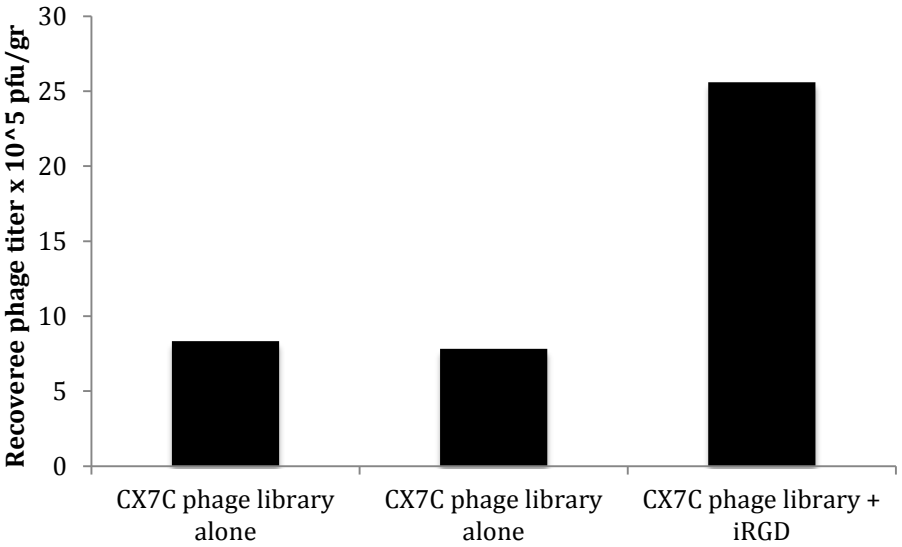


Figure 4. ***In vivo*** **penetration of CX_7C phage library into breast tumors.** Transgenic mice bearing breast tumors were injected with 5x10^9 pfu of phage expressing a CX7C peptide library enriched from cultured MDA-MB 468 cells. The phage were allowed to circulate for 30 minutes and tumor penetration was compared with or without a pre-injection of iRGD. Mice were perfused first with PBS, and then with an acid buffer for removal and inactivation of surface-bound phage. Penetrating phage were recovered by lysing the tumor tissues and further titered. iRGD pre-injection enhanced phage penetration by 3 fold.

Table 2. Peptide sequences from *in vivo* tumor screen using a library enriched by panning on cultured MDA-MB-468 cells*

1	CQKLPPRTC	24	CRGDRKGKC
2	CRGNDLKKC	25	CSDRRKRGC
3	CAGNGEAKC	26	CRGDRMGKC
4	CPPRGDMRRC	27	CVDELC
5	CPQTRSPRC	28	CKKRGDMPC
6	CTKRLRDSC	29	CRKRGDSPC
7	CVSRGDSLC	30	CVRLGGSDC
8	CKRGDKRAC	31	CTPSSANPC
9	CRGAKSTSC	32	CKKMSDRDC
10	CRGDKVPGC	33	CRSNPKKAC
11	CRGDKRGAC	34	CYRGDLGKC
12	CSGKLLSKC	35	CLSKR

13	CKRRGDTAC	36	CAKGQENPC
14	CRGDKVPGC	37	CRRNEKAPC
15	CLRDGRARC	38	CSGSRGTRC
16	CRSTGGSKC	39	CRGDRKTLC
17	CGPRRGDRC	40	CSRGDKGRC
18	CGRGDRQRC	41	CRRRGDSPC
19	CRTDLGS	42	CTRRRDTDC
20	CGRGDTKRC	43	CARLTGKAC
21	CARSR	44	CRGDKPDGC
22	CLRTKA	45	CRGDRKGKC
23	CSTGRRGDRC		

*The RGD/CendR motifs are prevalent in this *in vivo* screen as well.

Tumor macrophage (TAM) screens. We have developed methods for TAM isolation from 4T1 mouse breast cancer tumors and have tested our techniques with a previously identified peptide that strongly favors TAMs, although it also binds to tumor endothelial cells and tumor cells (Fogal et al., 2008; Hamzah et al., 2011).

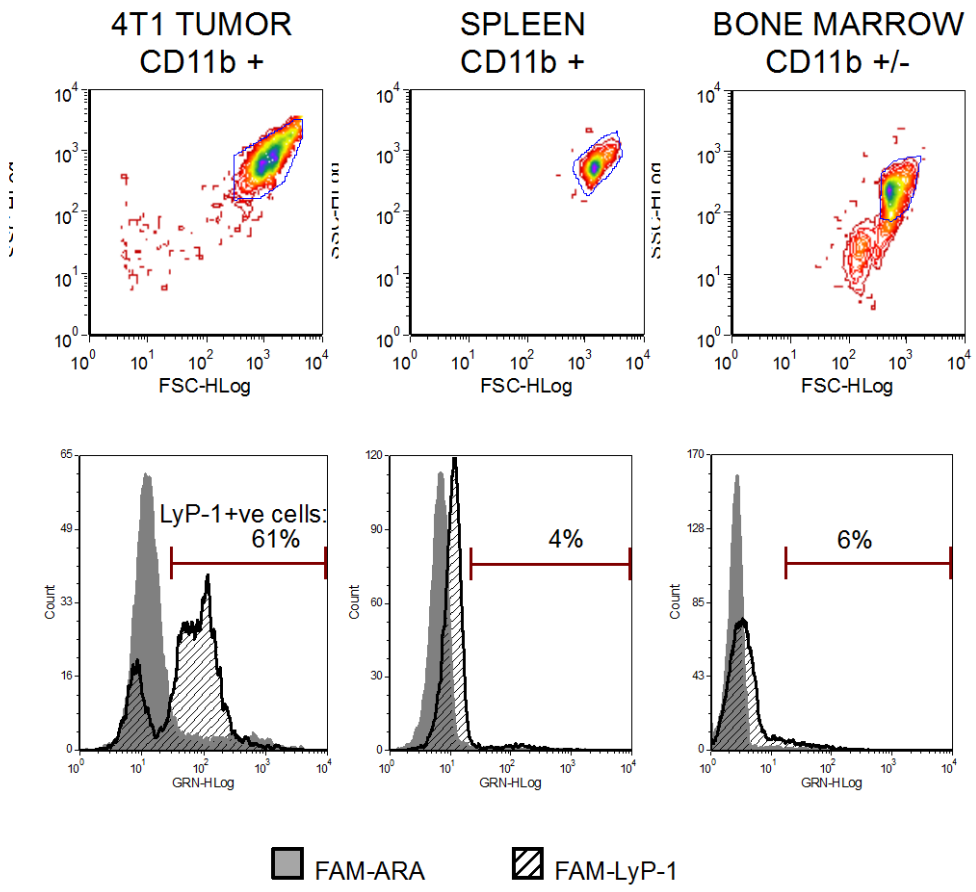


Figure 5. Isolation of tumor macrophages and demonstration of LyP-1 peptide binding in FACS.

We have used the 4T1 breast cancer model to isolate TAMs for *ex vivo* enrichment of phage clones from libraries. We dissociate the tumor tissues to single cells by incubation with collagenases I, and isolate the

macrophage population using magnetic microbeads coated with anti-CD11b antibody (Miltenyi Biotec). We have optimized the protocol for macrophage isolation (up to 10^7 CD11b+ cells from ~1cm^3 tumor) and shown by using LyP-1 peptide binding that the macrophages are positive for the binding of this peptide (Fig. 4). *Ex vivo* and *in vivo* phage display screening experiments using CD11b-positive cells from breast tumors are underway to discover peptides that are specific for tumor macrophages and that may detect a different macrophage subpopulation than LyP-1.

1b. *Perform tumor-penetrating screens for tumor-homing peptides in human breast cancer explants*

Ex vivo screening on fresh human breast tumor samples has been set up and looks promising; the phage accumulates 2 times more in the explants when the library is co-applied with iRGD (Fig. 2). Microscopy confirmed the penetration of phage co-incubated with iRGD into explants of several tumors. Both the phage and the peptide (which was labeled with fluorescein; FAM) showed efficient penetration into each of the explants tested, but not into normal breast tissue (Fig. 3). FAM-CRGDC, a non-tissue penetrating control peptide, did not facilitate phage penetration into the tumors.

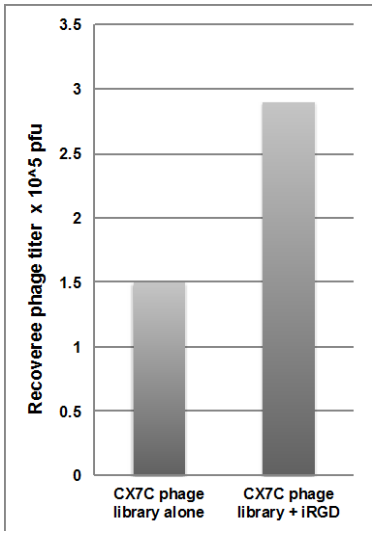


Figure 5. Penetration of CX_7C phage library into human breast tumor explants. Fresh human breast tumor samples were incubated with phage expressing a CX7C peptide library in the presence or absence of iRGD for 3 hours at 37˚C. Phage particles that bound to the surface of the explant were removed by acid wash, and only the phage that penetrated into the explant were recovered for titration. Note that iRGD facilitates phage penetration into the explant by 2 fold.

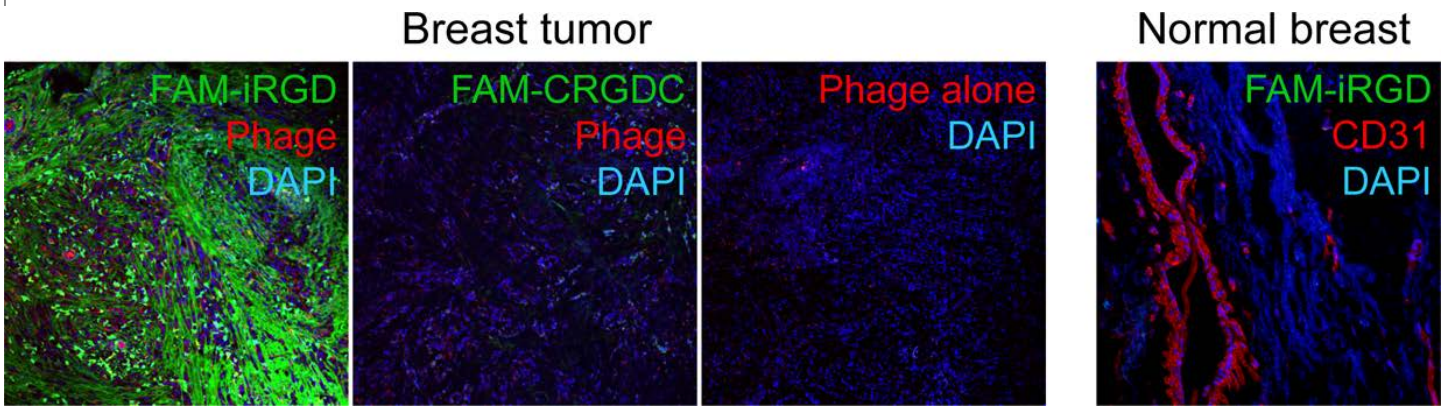


Figure 6. Co-penetration of iRGD and phage into human breast tumor explants. Fresh surgical samples of human breast tumors or normal breast tissue resected together with the tumors were maintained in short-term culture in the presence of FAM-iRGD (green) and phage expressing inert G_7 peptide for 90 min. The tumors were washed, fixed, and processed for confocal microscopy. Red, phage (except for the right most panel where red represents blood vessels); blue, nuclei.

Additional rounds of screening on the tumor explants are underway. An *in vivo* screen for CAFs in the MCF10CA1a tumor model has also been initiated recently in combination of iRGD.

1c. *Optimize and validate the experimental approach and custom-made bioinformatics software for high throughput phage sequencing*

Optimization of the HTS and bioinformatics is underway (Fig. 3). Briefly, phage DNA is purified, and subjected to emulsion PCR using primers with Ion Torrent adapters for clonal amplification on Ion Sphere Particles. The particles are isolated, loaded on a chip, and sequenced using an Iron Torrent next generation sequencer. A test run on a naïve phage library yielded highly diverse CX_7C peptide sequences. This method allows us to acquire peptide sequences of the entire phage pool to probe the full landscape of the homing peptides recovered. Thus, only a single round of selection is required for a screen to be informative, and eliminates any bias caused by differences in amplification rates of the phage clones.

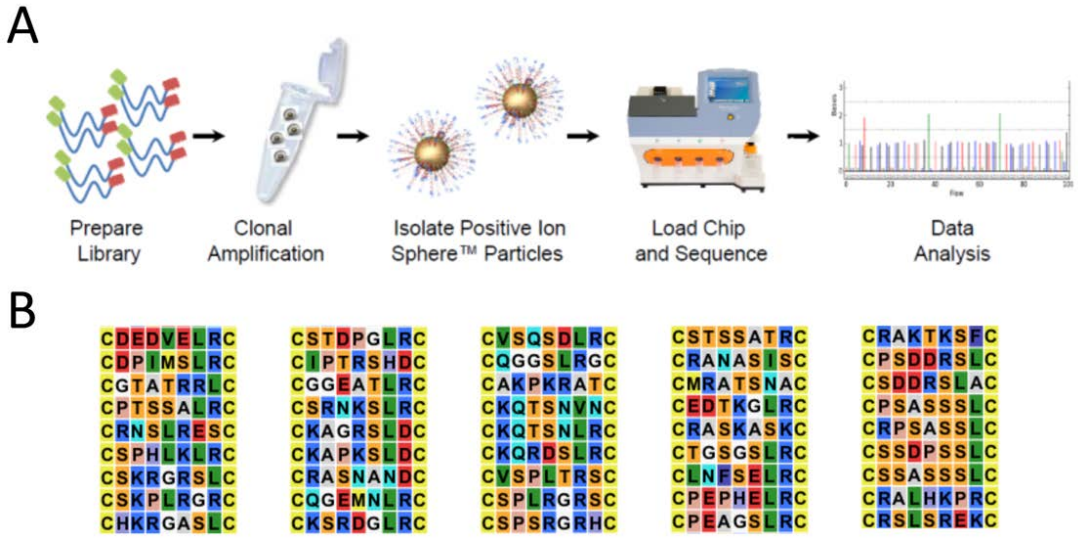
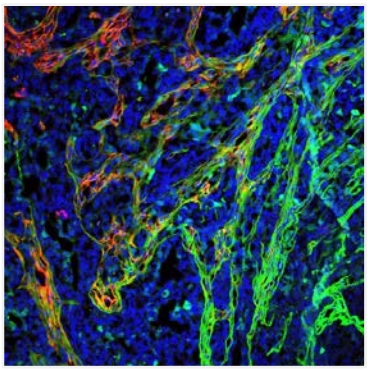


Figure 7. Phage DNA sequencing with Iron Torrent next generation sequencer. (A) Work flow of the sequencing procedure. After phage panning, phage particles are recovered for DNA purification. A DNA library is prepared using fusion primers with Ion Torrent adapters, and clonally amplified on Ion Sphere Particles by emulsion PCR. The particles are isolated, loaded on a chip, and subjected for sequencing with an Ion Torrent machine. (B) An example of peptide sequences in a naïve CX_7C library detected with Ion Torrent. Amino acids are color-coded based on their chemical characteristics.

Aim 2. ***To validate the homing specificities of individual phage and synthetic peptides from Task 1 in ex vivo and in vivo tests***



We have discovered that iRGD preferentially targets CAFs in breast tumors. Intravenously injected FAM-iRGD efficiently penetrated breast tumor stroma, and colocalized with CAFs (Fig. 7).

Figure 8. iRGD targets breast CAFs. Fluorescein (FAM)-labeled iRGD (green) was intravenously injected into mice bearing orthotopic MCF10CA1a human breast tumors, and was allowed to circulate for 30 min. The mice were perfused through the heart with PBS, and the tumors were subjected to immunofluorescence. Red, fibroblast marker (ER-TR7); Blue, nuclei. Note the co-localization of FAM-iRGD and fibroblasts.

Interestingly, iRGD detects fibroblasts in early pre-malignant breast cancer lesions in a transgenic model, before the blood vessels become positive for iRGD binding (Fig. 8), emphasizing the involvement of fibroblasts in tumorigenesis.

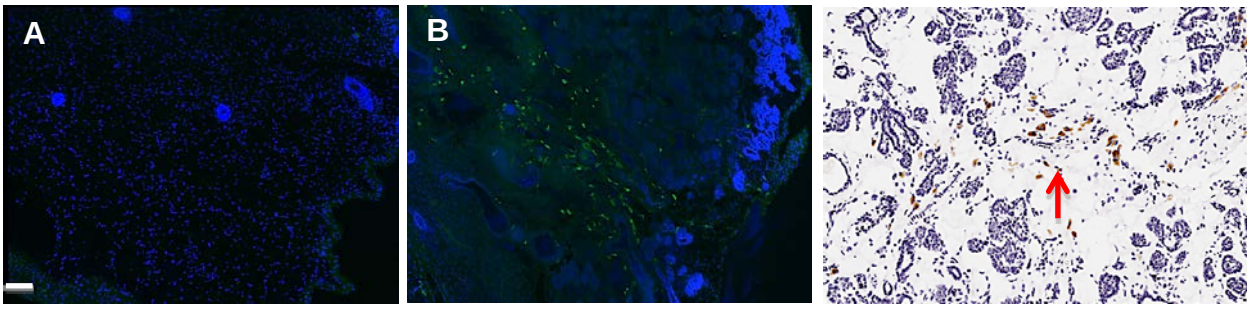


Figure 9. iRGD homes to early (premalignant) hyperplastic lesions in mammary fat pad isolated from MMTV-PyMT animals. Immunofluorescence on whole mount sections of mammary fat pad isolated following 1-hour circulation of 0.15 μmol FAM-iRGD in normal Blk6 mouse (**A)** or day 48 MMTV-PyMT mouse **(B**). Green – anti-FAM-iRGD; Blue - Nuclear Stain. Scale Bar -100 μm. **(C)** Anti-FAM staining in mammary fat pad sections isolated from an MMTV-PyMT mouse intravenously injected with FAM-iRGD.

Task 2b. *Analyze the homing specificity of homing peptides recognizing tumor-associated macrophages.*
Work on this aim has not been started.

Task 2c. *Analyze the homing specificity of homing peptides recognizing cancer stem cells*
Work on this aim has not been started.

3. KEY RESEARCH ACCOMPLISHMENTS

We have:

- Established tools and key technologies required for the proposed phage display project. These include mouse tumor models, human tumor explant model, immortalized human breast CAFs, cell lines enriched in breast cancer stem cells, CAF and TAM isolation techniques, and high throughput phage DNA sequencing.
- Demonstrated that phage co-administered with iRGD accumulates into tumors in vivo, and penetrates into into human breast tumor explants.
- Established an *in vivo* perfusion technique for focusing phage library screening to extravascular tumor tissue.
- Performed *in vitro*, *ex vivo*, and *in vivo* phage display screens using the new methods.
- Discovered that iRGD efficiently targets breast CAFs, and the iRGD receptor, NRP-1, is a potential CAF marker in breast tumors.

4. REPORTABLE OUTCOMES

Sanford-Burnham Institute has filed patent applications of the peptides described in this report, and on their receptors, when known.

5. CONCLUSIONS

We have established the infrastructure necessary for effective performance of the phage library screen designed to discover new peptides for the targeting of various cellular components in breast cancers. The CAF and breast cancer stem cell lines we have established and acquired, the CAF and TAM isolation techniques, and the high throughput sequencing of phage inserts will greatly facilitate the project. These tools have been applied to screen that have yielded promising results, making us confident that we will identify peptides specific for the targeted cell types in breast cancer during the remaining grant period. The unexpected discovery that iRGD, the peptide that we have been using to facilitate phage tumor penetration, is also an efficient CAF-targeting peptide, is a promising start in this regard. New probes for breast tumor detection and anti-breast cancer therapy should result from this work.

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Appendices: None